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(54) Title: ACTIVATED VITRONECTIN AS A MARKER OF ANGIOGENESIS DETECTED WITH PHAGE ANTIBODIES

(57) Abstract: The present invention provides a method for producing antibody fragments that discriminate between native and activated vitronectin, which can be used as markers of angiogenesis. Also provided are antibodies and peptides comprising said fragments, and therapeutic methods, particularly related to targeting for antibody-mediated antivascular cancer therapy and inhibiting the adhesion and migration of tumor-endothelial cells and platelets.

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Title: Activated vitronectin as a marker of angiogenesis
         detected with phage antibodies

FIELD OF THE INVENTION

The invention relates generally to medicine and the use
of antibodies. In particular the invention relates to
5   antibody fragments (and derivatives thereof) which have a
higher affinity for activated vitronectin than for native
vitronectin. Said fragments can be used as markers of
angiogenesis and other vitronectin-related diseases.

10   Antibodies are proteins that are produced by cells of
the immune system, so-called B lymphocytes, in response to
encounter with potentially harmful miro-organisms such as
bacteria and viruses. Antibodies are capable of binding to
the invading micro-organism that elicited their production
15   and aid in their elimination. This activity of the immune
system of producing antibodies in response to an invading
micro-organism has been exploited in the production of
monoclonal antibodies, a technology developed by Köhler and
Milstein (1975). Monoclonal antibodies are all those
20   immunoglobulin molecules that are produced by the progeny of
a single B lymphocyte. Conventionally, monoclonal antibodies
are obtained by immunizing a mouse with an antigen and fusing
the spleen or lymphnode B lymphocytes with an immortalized
murine plasma cell line. The ensuing hybrid cell lines
25   (hybridomas) bear the characteristics of both parental cell
types: they are immortal and produce a single species of
monoclonal antibody specific for the antigen used to immunize
the mouse. The advantage of monoclonal antibodies is that
they represent a homogeneous population of immunoglobulin
30   molecules with a pre-defined binding specificity. Over the
years, monoclonal antibodies have proven invaluable tools in
research and diagnostics.

Soon after the invention of hybridoma technology, the enormous potential of monoclonal antibodies in human therapy was realized. Because of their high binding specificity, antibodies were hypothesized, among others, to be capable of

5   binding to viruses and bacteria and their toxic products facilitating their elimination. In other applications, monoclonal antibodies were envisaged to specifically bind to tumor cells to promote their eradication or to bind to soluble molecules produced by cells of the immune system to

10   neutralize their activity in harmful chronic inflammatory conditions and/or in autoimmune disease. Indeed, monoclonal antibodies have been described as magic bullets that could be used in the treatment of a wide variety of human diseases.

15   Numerous clinical studies with monoclonal antibodies of non-human, usually murine origin showed that they performed poorly as a result of their immunogenicity; upon injection of murine antibodies in humans, the human immune systems recognizes the murine antibodies as foreign proteins

20   resulting in the induction of an immune response against the murine protein. In addition, murine antibodies have poor pharmacokinetic properties in humans and are inefficient in recruiting effector functions of cells of the immune system. These issues have spurred the development of alternative

25   strategies to obtain more human antibodies for therapy.

In one approach of creating more human antibodies, the immunoglobulin variable regions encoding murine antibodies are genetically used to human immunoglobulin constant

30   regions. The resulting chimeric antibody still contains >30% murine amino acid sequences. Clinical application in humans of chimeric monoclonal antibodies has shown that these proteins retain their immunogenicity in the majority of cases. In another approach, only immunoglobulin variable

35   region sequences relevant for antibody specificity are of murine origin; the constant regions of the immunoglobulin

molecule as well as the framework regions of the variable
region are of human origin. Clinical application of these
"humanized" monoclonal antibodies indicates that these
molecules are generally more effective and have no or little
5   intrinsic toxicity or immunogenicity. However, reconstructing
the original affinity and specificity in a humanized version
of a murine antibody is a time-consuming process and may
render the antibody less human. These considerations with
chimeric and humanized monoclonal antibodies and the recent
10   clinical success of engineered monoclonal antibodies spurred
the development of efficient methods for the isolation and
production of fully human monoclonal antibodies, the most
desirable antibody format for clinical application.

15   One method of obtaining human monoclonal antibodies
employs transgenic mice harboring human immunoglobulin loci
in combination with conventional hybridoma technology. In
these mice, large portions of human immunoglobulin heavy and
light chain loci have been inserted in the mouse germ line
20   while the endogenous murine immunoglobulin loci have been
silenced by gene knockout. Immunization of these transgenic
mice with an antigen results in the production of human
antibodies specific for the antigen. Human monoclonal
antibody-producing cell lines can be obtained from these mice
25   by fusing the spleen cells of immunized mice with plasma cell
lines in vitro to obtain immortalized monoclonal antibody-
secreting hybridomas. Importantly, production of human
antibodies in transgenic mice depends on immunization
procedures and is governed by constraints of the murine
30   immune response. As a consequence, it is difficult if not
impossible to obtain antibodies against the mouse's own
antigens (autoantigens), to xenoantigens that have a high
degree of homology to murine autoantigens or to antigens that
have poor immunogenic properties such as polysaccharides.
35   These notions have spurred the development of molecular
approaches that obviate the need for immunization and cell

immortalisation to obtain human monoclonal antibodies with
desired specificities. These strategies are based on
"immortalisation" of the immunoglobulin genes encoding the
antibodies rather than the cell lines producing them.

5

An alternative method of obtaining human monoclonal
antibodies with desirable binding properties employs phage
display libraries, an *in vitro*, recombinant DNA-based
approach that mimics key features of the humoral immune
10   response. For the construction of phage display libraries,
collections of human antibody heavy and light chain variable
region genes are expressed on the surface of bacteriophage
particles, either in single chain Fv (scFv) or in Fab format.
Large libraries of antibody fragment-expressing phages
15   typically contain > 10^9 antibody specificities and may be
assembled from the immunoglobulin V regions expressed in the
B lymphocytes of immunized or non-immunized individuals.
Alternatively, phage display libraries may be constructed
from immunoglobulin variable regions that have been partially
20   assembled in vitro to introduce additional antibody diversity
in the library ("semi-synthetic" libraries). For example, *in
vitro* assembled variable regions contain stretches of
synthetically-produced, randomized or partially-randomized
DNA in those regions of the molecules that are important for
25   antibody specificity. Thus, specificities not present in
natural repertoires may be added to phage display libraries
of antibody fragments.

Recombinant phages expressing antibody fragments of
30   desirable specificities may be selected from a library by one
of several methods. Target antigens are immobilized on a
solid phase and subsequently exposed to a phage library to
allow binding of phages expressing antibody fragments
specific for the solid phase-bound antigen. Non-bound phages
35   are removed by washing and bound phages eluted from the solid
phase for infection of *Escherichia Coli* (*E.coli*) bacteria and

subsequent propagation. Multiple rounds of selection and propagation are usually required to sufficiently enrich for phages binding specifically to the target antigen. Phages may also be selected for binding to complex antigens such as

5   complex mixtures of proteins or whole cells. Selection of antibodies on whole cells has the advantage that target antigens are presented in their native configuration, unperturbed by conformational changes that are introduced by immobilizing an antigen to a solid phase. The constraints

10   imposed by the natural immune response and the influence of the immunogenicity of the target antigen does not permit the isolation of monoclonal antibodies against any antigen by conventional hybridoma technology. In phage approaches, these factors do not play a role, allowing the isolation of

15   monoclonal antibodies against "difficult" antigens such as autoantigens, carbohydrates and toxic antigens.

In one particular selection procedure, phage display libraries are used in combination with flow cytometry and

20   cell sorting to isolate antibody fragments against molecules expressed on the plasma membrane of subpopulations of eukaryotic cells present in a heterogeneous mixture. Thus, the heterogeneous mixture of cells is incubated with the phage library allowing phages to bind to the different cell

25   types. Subsequently, the cells are stained with fluorochrome-labeled monoclonal antibodies to permit identification of the subpopulation of target cells by immunofluorescence analysis and flow cytometry. Target cells and attached phages are collected by flow cytometry and the attached phages are

30   eluted and propagated. This method is rapid, independent of the immunogenicity of the target antigen and yields antibody fragments against molecules in their native configuration. Specific antibodies against very small populations of cells in a heterogeneous mixture can be obtained.

35   For production of intact human monoclonal antibodies, scFv with desirable specificities can be inserted into

mammalian expression vectors containing the genes encoding human immunoglobulin constant regions. We have recently developed a series of constructs that permit the rapid conversion of phage display library-derived scFv antibody

5   fragments to fully human monoclonal antibodys of each immunoglobulin isotype and subclass. Transfected cell lines harboring these constructs produce human monoclonal antibodies *in vitro* that are correctly assembled and glycosylated.

10

Treatment with monoclonal antibodies or antibody-derivatives directed against tumor surface antigens has proved to be a rational strategy in tumor immunotherapy. Immunotherapy in cancer is aimed at recruiting the humoral

15   and/or cellular arms of the immune system to eradicate tumor cells. To that end, a variety of approaches has been tested in *in vivo* and *in vitro* systems including unconjugated antibodies, bi-specific antibodies, immunotoxins, radiolabeled monoclonal antibodies, immunoliposomes and

20   cytotoxic T lymphocytes. For example, treatment of a large cohort of patients with resected Dukes C colorectal cancer with a murine antibody against the Ep-CAM molecule expressed on colorectal tumor cells has proved very effective. After 7 years of follow-up evaluation, overall mortality of patients

25   was reduced by 32% and the recurrence rate was decreased by 23%. In another example, a humanized antibody against the HER2/neu receptor that is over-expressed on the tumor cells of 25-30% of patients with breast cancer, was shown to slow the progression of tumor growth and to increase the

30   percentage of patients who experienced tumor shrinkage. In another example, a chimeric monoclonal antibody against the CD20 antigen was shown to give a response in 48% of patients with relapsed low grade or follicular lymphoma.

In the above-mentioned examples, treatment of patients

35   with the monoclonal antibody is sufficient for clinical effect. Binding of the antibody to the target antigen results

in the recruitment of components of the complement system and
effector cells of the immune system with Fc receptor for
antibody constant regions that act in concert to kill the
tumor cell. For reasons unknown, binding of an antibody to a
5   target antigen on the membrane of a cell does not always
result in tumor cell killing. For some antigens it is
necessary to arm the monoclonal antibody with entities that
kill the tumor cells via other mechanisms such as toxins or
radioactive molecules.

10   In these approaches the specificity of the antibody
employed is crucial; ideally, the antibody should
specifically bind to tumor cells with minimal cross-
reactivity with normal tissues. This criterium is not always
met as illustrated by the chimeric anti-CD20 monoclonal
15   antibody. The CD20 antigen is expressed by B cell tumors but
also by non-malignant immature and mature B lymphocytes.
Despite this cross-reactivity with non-malignant B cells,
treatment with the chimeric anti-CD20 antibody causes few
side-effects and the clinical success is considerable.
20

         The extracellular matrix controls cell survival, cell
morphology and tissue organization by supporting cell
adhesion. Remodeling of the extracellular matrix is a key
process in the development of properly organised blood
25   vessels, tissues and organs. Many extracellular matrix
proteins, such as fibronectin and members of the collagen
family, and proteases, such as plasmin and
metalloproteinases, are involved in the remodeling process.
At the cellular level, remodeling is regulated by members of
30   the "integrin" family of adhesion receptors, which use the
extracellular matrix proteins as attachment sites for
migration.
         A large number of studies have indicated that matrix
remodeling and cell migration also play a key role during
35   pathological processes (Werb 1997; Liotta et al. 1991). For
example, tumor cell invasion into tissues is dependent on

matrix proteolysis. Inhibition of proteases, such as
urokinase-type plasminogen activator ("uPA") or
metalloproteinases, prevent the formation of metastases
(Jankun et al. 1997; Min et al. 1996; Ignar et al. 1998).
5   Matrix remodeling and integrin dependent migration are also
crucial for angiogenesis, the formation of new blood vessels
(Friedlander et al. 1995). The integrins αvβ3 and αvβ5, in
particular, play an important role during angiogenesis
(Brooks et al. 1994a). Their expression is elevated on
10 angiogenic endothelial cells and antagonists of αvβ3 suppress
tumor growth and can induce tumor regression.

        Vitronectin is an important extracellular matrix
molecule implicated in the control of cell morphology,
proliferation, migration and survival. Because vitronectin is
15 the most important adhesive protein in serum, which is used
to culture cells in vitro, vitronectin is also called "serum
spreading factor". Vitronectin is the primary ligand for αvβ3
and αvβ5 integrins, and plays a prominent role in
angiogenesis and tumor growth (Preissner et al. 1997 and
20 1998). Vitronectin is a 75 kDa glycoprotein that is
synthesized primarily in the liver, and is present in plasma
in a concentration between 200-400 mg/L (for review see
Preissner et al. 1998; Seiffert 1997; Schvartz et al. 1999).
In plasma, vitronectin is present in a "native" conformation,
25 whereas in serum, vitronectin is present in its "activated"
conformation. Activation of vitronectin can be mediated by a
complex of thrombin-antithrombin III and occurs during
pathological processes, including angiogenesis. When
hyperpermeability is induced by angiogenic factors such as
30 vascular endothelial growth factor ("VEGF"), vitronectin is
deposited into the extracellular matrix and forms part of a
provisional matrix.

        Besides its adhesive function, activated vitronectin
binds, concentrates, and regulates proteins that control the
35 formation of the serine protease plasmin. These proteins
include plasminogen, plasminogen activator inhibitor type

1(PAI-1) and the urokinase-type plasminogen activator
receptor (uPAR, Kost et al. 1992). By providing a link
between plasmin regulated matrix proteolysis and integrin-
mediated migration, activated vitronectin appears to play a
5   central role in extracellular matrix remodeling and diseases.
        Although several immunohistochemical studies have
demonstrated the presence of vitronectin in both normal
tissues and in tissues of different pathological origin, the
role of activated vitronectin to date has been unclear
10   (Sumiyoshi et al. 1991; Carreiras et al. 1996; Hayman et al.
1983; Loridon-Rosa et al. 1988; Reilly and Nash 1988;
Rosenblum and Carsons 1996; Sobel et al. 1995; Sumida et al.
1990; Weller et al. 1991). Studies on the role of activated
vitronectin have thus far been hampered by the lack of
15   antibodies that can discriminate between native and activated
vitronectin which could, accordingly, be used in immuno-
histochemical analysis.

In the present invention specific (single chain) antibody fragments, and whole antibodies comprising said fragments, were generated against activated vitronectin using phage display technology. These (scFv) antibody fragments and

5   antibodies were used to determine the role of activated vitronectin in several pathological processes, including tumor growth. Using these fragments and antibodies, the presence and localization of activated vitronectin in various pathological conditions was analyzed. Whereas, in normal

10   tissues, activated vitronectin could hardly be detected, activated vitronectin was abundantly found in the extracellular matrix of various tumors, and in sclerotic kidney as well as in acutely inflamed lung tissue. Activated vitronectin was primarily localized around blood vessels, in

15   large fibrous tissue and in plaques. These results indicate that the activated form of vitronectin is almost exclusively present in disease sites, in particular disease sites where angiogenesis is involved.

Accordingly, the invention includes the specific (single

20   chain) antibody fragments themselves, the corresponding antibodies containing these fragments, and means and methods for making the antibodies and utilizing the antibodies and fragments in the diagnosis and treatment of disease. Diseases to be treated include all diseases involving deposition or

25   presence of activated vitronectin, such as psoriasis, rheumatoid arthritis, artherosclerosis and malignancies. The invention further includes a kit of parts that includes the antibodies or their active fragments.

In one aspect, the invention includes a method of tumor

30   therapy in a subject. The method involves administering to the subject anti-activated vitronectin antibodies and/or antibody fragments which interfere with the adhesion and/or migration of tumor-endothelial cells by preventing vitronectin binding to its receptor, thus providing an

35   antibody-mediated, anti-vascular cancer therapy.

Vitronectin is an important adhesive molecule and, in
its activated state as a component of the extracellular
matrix, a potential target for tumor therapy. Previous
immunohistochemical studies have demonstrated the presence of
5   substantial deposits of vitronectin in most tumors examined
and in a number of inflammatory disease states (Carreiras et
al. 1996; Loridon-Rosa et al. 1988; Dahlback et al. 1988;
Tomasini-Johansson et al. 1998; Guettier et al. 1989; Gladson
and Cheresh 1991; Jaskiewicz et al. 1993; Jang et al. 1998).
10   Importantly, however, these earlier studies were performed
with antibodies which could not discriminate between native
and activated vitronectin. As established herein, to assess
the role of vitronectin in angiogenesis, it is important to
distinguish between the activated form and the circulating,
15   native form of vitronectin.

By performing subtractive phage selections on urea-
activated vitronectin in the presence of plasma as source of
native vitronectin, we obtained scFv antibody fragments that
discriminated between the two forms of vitronectin. Thus,
20   phage display proved to be a powerful tool to generate scFv
antibody fragments that discriminate between the activated
and inactivated form of a protein.

We extend the finding that vitronectin deposits are
found in most tumors examined and in a number of inflammatory
25   diseases by demonstrating that these deposits are indeed
formed by activated vitronectin. The importance of activated
vitronectin as a provisional matrix was supported by the
virtual absence of activated vitronectin in normal tissue.
Of note, activated vitronectin was not exclusively localised
30   in the surroundings of blood vessels, but was present in
extra-cellular deposits throughout areas in the tumors and in
similar regions in focal glomerular sclerosis and lung tissue
from a patient with pneumonia.

Although the precise role of vitronectin in these areas
35   has not yet been fully elucidated, vitronectin is thought to
play an important role in the adhesion of (tumor)-endothelial

cells to a provisional matrix (Carreiras et al. 1999a) and
concentrating proteolytic activity on the cell surface and
extracellular matrix by trapping key components, like uPA,
uPAR, PAI-1 and plasminogen (Waltz et al. 1997). Furthermore,
5   vitronectin decreases microvascular endothelial cell and
tumor cell apoptosis (Isik et al. 1998; Uhm et al. 1999).
Thus, activated vitronectin appears to be prominently
localized at sites where angiogenesis and tumor cell
migration is thought to play a crucial role in tumor growth.
10   Moreover, the fact that vitronectin is normally mainly
produced by the liver and that several tumor cell lines also
produce vitronectin further indicates that vitronectin plays
a role in these conditions (Carreiras et al. 1999b; Tomasini-
Johansson et al. 1994; Yasumitsu et al. 1993). Finally
15   platelets, which also contain activated vitronectin (Seiffert
and Schleef 1996) and are believed to play an important role
in angiogenesis and tumor growth, further stresses an
important role for activated vitronectin in pathological
processes (Pinedo et al. 1998). Activated vitronectin is the
20   ligand for the $\alpha v \beta 3$ integrin, which is upregulated during the
angiogenic process and plays an important role in adhesive
interactions during angiogenesis (Brooks et al. 1994b).
Inhibiting monoclonal antibodies against molecules involved
in the adhesion and migration of tumor-endothelial cells have
25   showed promising results in treating tumor-bearing mice
(Brooks et al. 1995; Kobayashi et al. 1994; Tsuchiya et al.
1995). Because activated vitronectin binds, concentrates and
regulates proteases like plasminogen, PAI-1 and uPAR (Kost et
al. 1992), it may be predicted that antibodies specific for
30   activated vitronectin prevent the binding of these proteases
thereby inhibiting proteolysis and the invasive potential of
endothelial cells. Extrapolating from these results, it may
be anticipated that by preventing vitronectin from binding to
its receptor a similar result can be obtained. This renders
35   the activated form of vitronectin a potential target for
antibody-mediated anti-vascular cancer therapy.

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In one aspect the invention provides an antibody fragment comprising at least a CDR3 domain of a heavy chain of an antibody which has a higher affinity for activated
5   vitronectin than for native vitronectin.

Preferably, said antibody fragment is a human or human-like antibody fragment. This renders said fragment more effective and lowers the toxicity or immunogenicity. It is indicated that human or human-like antibody fragments have no
10   or little immunogenicity at all.

More preferably, said antibody fragment is obtainable by subtractive phage selections on urea-activated vitronectin in the presence of plasma as a source of native vitronectin. In one embodiment an antibody fragment of the invention is
15   provided, having a V-region comprising an amino acid sequence of SEQ. I.D. NO. 1, SEQ. I.D. NO. 2, SEQ. I.D. NO. 3, SEQ. I.D. NO. 4 or a functional part, derivative and/or analogue thereof. Of course, one or more amino acid substitutions can be made in the above mentioned sequences, while retaining at
20   least one property of said sequences in kind, not necessarily in amount. It is known to a person skilled in the art, that a substitution of one amino acid with another amino acid with generally similar properties (like size and hydrophobicity) in a certain amino acid sequence does not alter much of the
25   overall functioning of said amino acid sequence. This is called conservative substitution. For instance, a glycine residue may be substituted with an alanine residue. Thus, the invention also provides an antibody fragment, comprising at least 60 percent, more preferably 75 percent, more preferably
30   85 percent, most preferably 95 percent homology to an amino acid sequence of SEQ. I.D. NO. 1, SEQ. I.D. NO. 2, SEQ. I.D. NO. 3 , SEQ. I.D. NO. 4 or a functional part, derivative and/or analogue thereof.

An antibody fragment of the invention is preferably
35   directed against human activated vitronectin. However, said antibody fragment is also capable of binding to activated

vitronectin of other species, as for instance chicken activated vitronectin (example 9).

By an antibody fragment is meant herein a fragment of at least the size of a CDR3 domain, in particular a fragment
5   derived from a CDR3 of a heavy chain. This fragment has at least one same property in kind, not necessarily in amount, as said antibody.

A higher affinity of a fragment for activated vitronectin than for native vitronectin means herein that
10 said fragment binds more activated vitronectin than native vitronectin when said fragment is exposed to the same excess amount of both kinds of vitronectin. This can be measured in a mixture, comprising equal excess amounts of activated and native vitronectin. This mixture can be provided to a certain
15 amount of the same kind of antibody fragments. After incubation it is possible to determine the amount of bound activated vitronectin and bound native vitronectin. If it is then concluded that said antibody fragments have bound more activated vitronectin than native vitronectin, said antibody
20 fragments are considered to have a higher affinity for activated vitronectin than for native vitronectin.

A functional part of a protein, for instance a functional part of an antibody, is defined herein as a part which has the same kind of properties in kind, not
25 necessarily in amount. Said properties may for instance comprise the capability to bind to a specific antigen. A functional derivative of a protein is defined as a protein which has been altered such that the properties of said molecule are essentially the same in kind, not necessarily in
30 amount. A derivative can be provided in many ways, for instance through conservative amino acid substitution. For instance, one or more glycine residues may be substituted for alanine, which will not alter much of the properties of said protein.
35   A person skilled in the art is well able to generate analogous compounds of a protein, for instance an antibody.

This can for instance be done through screening of a peptide library. Such an analogue has essentially the same properties, for instance binding properties, of said protein in kind, not necessarily in amount.

Further provided is an antibody comprising an antibody fragment of the invention. Preferably, said antibody is suitable for being provided to the blood circulation of an individual. In that case, a target molecule for said antibody can be reached via the blood circulation. Of course, said antibody must be sufficiently stable to remain in the blood circulation until said target molecule is reached. As is known to a person skilled in the art, IgG antibodies are very suitable for providing to the blood circulation of a subject. Thus, in a preferred aspect the invention provides an antibody of the invention which is an IgG antibody. The construction and production of an IgG_1 antibody comprising an antibody fragment of the invention is described in example 4. An antibody or fragment of the invention can, for instance, be used to diagnose a disease state in a subject, for instance through detecting the presence of activated vitronectin in a biological sample taken from said subject.

In another aspect the invention provides a pharmaceutical composition comprising an antibody fragment according to the invention. Such composition can be used in an anti-vascular therapy, preferably, a cancer therapy. The invention therefore also provides the use of an activated form of vitronectin as a target for antibody-mediated anti-vascular cancer therapy. In one embodiment a method of cancer therapy is provided, comprising administering anti-activated vitronectin antibody fragments or antibodies according to the invention, which interfere with the adhesion and/or migration of tumor-endothelial cells, to a patient.

Now that a role of activated vitronectin in the development of certain diseases, for instance cancer, is

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established, activated vitronectin can be used as a marker to
detect said diseases. Thus, in one aspect the invention
provides a method of detecting the presence of activated
vitronectin comprising using an antibody fragment or an
5   antibody of the invention. The use of an antibody fragment or
an antibody of the invention for the detection of the
presence of activated vitronectin is also herewith provided.
       In another aspect the invention provides a method of
diagnosing a disease state in a subject, said method
10   comprising detecting for the presence of activated
vitronectin in a biological sample taken from said subject.
Preferably, said activated vitronectin is detected with use
of an antibody and/or an antibody fragment, more preferably
with use of an antibody and/or an antibody fragment of the
15   invention. In one embodiment said detection of activated
vitronectin comprises detecting the presence of said
antibody, antibody fragment, or a part thereof, bound to said
vitronectin. A lot of techniques are known in the art for
detecting the presence of an antibody, for instance bound to
20   activated vitronectin. Said antibody may for instance be
labeled with a fluorescent or radioactive label. After said
antibody has been allowed to bind to activated vitronectin,
unbound antibody can be removed by one or more washing steps.
After that, a fluorescent or radioactive signal will reveal
25   the presence of bound antibody and, hence, the presence of
activated vitronectin. Of course, a person skilled in the art
can think of many alternative ways to detect the presence of
an antibody, for instance bound to activated vitronectin.

30   Now that it is shown to be possible to detect an
activated form of vitronectin, a person skilled in the art is
put in the position that he/she is able to devise strategies
for finding additional binding molecules specific for
activated vitronectin using means and methods available in
35   the art. The invention therefore also provides a method of
manufacturing an antibody or an antibody fragment directed

toward detecting the presence of activated vitronectin. Such
method can include the use of an apparatus for detecting the
presence of activated vitronectin, derived by selection from
a library using a phage display method. The invention

5   therefore also provides such an apparatus. In another
embodiment the invention provides an apparatus for detecting
the presence of activated vitronectin, using an antibody or
an antibody fragment, said antibody or fragment derived by
selection from a library comprising urea-treated vitronectin

10   capable of binding to an antibody or antibody fragment of
interest, such binding forming a receptor complex and thereby
causing a change in a property associated with the binding
partner; and a detection means for detecting the measurable
change. Preferably, the receptor complex comprises an

15   antibody or an antibody-binding fragment thereof, a peptide
or a nucleotide sequence. In another preferred embodiment the
receptor complex comprises an antibody or an antibody-binding
fragment thereof derived by selection from a library using a
phage display method.

20

The invention further provides an isolated peptide
fragment comprising at least a CDR3 domain of a heavy chain
of an antibody which has a higher affinity for activated
vitronectin than for native vitronectin. Preferably, said

25   peptide fragment is obtainable by subtractive phage
selections on urea-activated vitronectin in the presence of
plasma as a source of native vitronectin. Mutants of a
peptide of the invention can be easily tested for the same
binding characteristics in kind, not necessarily in amount,

30   and are therefor also part of the invention.

Activated vitronectin is the most important adhesive
protein in serum. Binding of an antibody fragment of the
invention to said vitronectin will therefore affect the

35   capability of said vitronectin to bind to a lot of different
targets. Said target may for instance be an endothelial cell,

preferably a tumor endothelial cell, but may also for
instance be platelets. Thus, in one aspect the invention
provides a use of an antibody or an antibody fragment
according to the invention for at least in part changing an
5   adhesion property of activated vitronectin.
       Said adhesion property preferably comprises the
capability to bind to endothelial cells, extracellular matrix
proteins, and/or platelets. In a more preferred embodiment,
said changing of the capability to bind to extracellular
10   matrix proteins prevents angiogenesis.

       Now it is possible, using an antibody fragment of the
invention, to at least in part change the capability of
activated vitronectin to bind to endothelial cells and/or
15   collagen. Thus one embodiment of the invention provides a
method for at least in part inhibiting adhesion of an
endothelial cell to activated vitronectin, comprising
providing said vitronectin with an antibody fragment, an
antibody or a peptide according to the invention.
20   Another embodiment of the invention provides a method
for at least in part decreasing an interaction of activated
vitronectin with collagen and/or endothelial cells,
comprising providing said vitronectin with an antibody
fragment, an antibody or a peptide according to the
25   invention.

       Another property of activated vitronectin is it's
capability to decrease microvascular endothelial cell and
tumor cell apoptosis (Isik et al. 1998; Uhm et al. 1999).
30   This can lead to unlimited cell growth and division, which
leads for instance to tumor development and/or angiogenesis.
This highly undesirable effect of activated vitronectin to
decrease cell death can now be counteracted by an antibody
fragment, an antibody and/or a peptide of the invention.
35   Thus, in one aspect the invention provides a method for at
least in part inducing cell death of an endothelial cell,

associated with an extracellular matrix comprising activated
vitronectin, comprising providing said matrix with an
antibody fragment, an antibody or a peptide according to the
invention. Said cell death may comprise apoptosis.

5

In yet another aspect the invention provides an isolated
peptide comprising a vitronectin epitope which is recognized
by an antibody fragment, an antibody or a peptide according
to the invention. Said peptide may for instance be useful for
10   screening a library for their binding properties to said
epitope and, hence, for activated vitronectin. As is
described in example 8, the vitronectin H2 domain is an
important epitope for an antibody fragment of the invention.
Thus, in a preferred aspect the invention provides a peptide
15   according to the invention, which comprises at least part of
the vitronectin H2 domain.

Now that an antibody fragment of the invention is
provided, a person skilled in the art is well capable of
20   generating a nucleic acid molecule encoding said antibody
fragment. Methods to generate nucleic acid molecules encoding
a certain polypeptide, starting from the amino acid sequence
of said polypeptide, are known in the art, as well as codon
usage in different organisms. Hence this needs no further
25   explanation here. Thus the invention provides an isolated or
recombinant nucleic acid molecule encoding an antibody
fragment, an antibody or a peptide according to the
invention, or a functional part, derivative and/or analogue
thereof.

30   Of course, a person skilled in the art is equally well
capable of generating a nucleic acid molecule encoding a
peptide comprising a vitronectin epitope which is recognized
by an antibody fragment, an antibody or a peptide according
to the invention, or a functional part, derivative and/or
35   analogue thereof. Said nucleic acid molecule is also herewith
provided.

20

Preferably, said epitope comprises at least part of the
vitronectin H2 domain. With methods known in the art the
artisan is also capable of generating a fusion gene
comprising a nucleic acid molecule of the invention. Said
5   fusion gene may encode a fusion protein. Thus, in one aspect
the invention provides a fusion protein comprising a peptide
encoded by a nucleic acid molecule of the invention.

By at least a functional part of a nucleic acid of the
10 invention is meant a part of said nucleic acid, at least 30
base pairs long, preferably at least 200 base pairs long,
comprising at least one expression characteristic (in kind
not necessarily in amount) as a nucleic acid of the
invention.

15

During the process of angiogenesis and tumor growth,
platelets, which contain activated vitronectin, are also
believed to play an important role. Therefore said platelets
are also an important target for antibody-mediated anti-
20 vascular cancer therapy. As is described in example 10, an
antibody fragment of the invention is able to reduce the
activation of platelets. Thus one embodiment of the invention
provides a method for at least in part inhibiting platelet
aggregation in the presence of vitronectin, comprising
25 providing said vitronectin with an antibody fragment, an
antibody or a peptide of the invention.

With the teachings of the present invention it is clear
that an antibody fragment, an antibody or a peptide of the
30 invention is very suitable for the preparation of a
medicament. A use of an antibody fragment, an antibody or a
peptide of the invention for the preparation of a medicament
is therefore also provided. Of course, said medicament is
particularly suited for at least one of the above mentioned
35 applications. Thus, in one preferred aspect, a use of an
antibody fragment, an antibody or a peptide of the invention
for the preparation of a medicament for at least in part

inhibiting platelet aggregation is provided. In another
preferred aspect, a use of an antibody fragment, an antibody
or a peptide of the invention for the preparation of a
medicament for at least in part decreasing an interaction of
5   activated vitronectin with collagen and/or endothelial cells
is provided. Another preferred embodiment provides a use of
an antibody fragment, an antibody or a peptide of the
invention for the preparation of a medicament for at least in
part changing the development of blood vessels.
10

    Activated vitronectin is an important extracellular
matrix protein implicated in the control of cell morphology,
proliferation, migration and survival. Activated vitronectin
is for instance involved in the development of blood vessels.
15   With the teaching of the present invention it is possible to
change said development of blood vessels. This can be done by
influencing the remodeling process of the extracellular
matrix of an endothelial cell. Thus, one embodiment provides
a method for at least in part changing the development of
20   blood vessels, comprising influencing the remodeling process
of the extracellular matrix of an endothelial cell. In a
preferred embodiment, said extracellular matrix is provided
with an antibody fragment, an antibody or a peptide according
to the invention.
25   Now that an antibody fragment with a higher affinity for
activated vitronectin than for native vitronectin is
provided, the artisan is well capable of designing an
antibody fragment, preferably based on a fragment of the
invention, with a same binding property in kind, not
30   necessarily in amount. Such fragment is capable of at least
in part inhibiting binding of an antibody fragment, an
antibody or a peptide of the invention, to activated
vitronectin. By at least in part inhibiting binding of a
molecule to activated vitronectin is meant herein that less
35   molecules will bind to a certain limiting amount of activated
vitronectin if an inhibitor is also provided. If a limiting

amount of activated vitronectin is provided with a certain
amount of said molecules and an inhibitor, at least a part of
said inhibitor will bind to said activated vitronectin. The
inhibitor-bound activated vitronectin is not capable of
5   binding to said molecules anymore. This results in less
amount of said molecules to be bound to vitronectin, as
compared to a situation wherein no inhibitor was provided.

Thus, in one aspect, the present invention provides an
antibody or functional part, derivative and/or analogue
10 thereof, capable of at least in part inhibiting binding of an
antibody fragment, an antibody or a peptide of the invention,
to activated vitronectin.

MATERIALS AND METHODS

A phage antibody library cloned in the phagemid pPVT
with a size of 4.5 x 10⁵ specificities was used. Urea treated
5   vitronectin was prepared as described earlier (Yatohgo et al.
1988). Native vitronectin was a gift from Dr K.T. Preissner
(Bad Nauheim, Germany). The rabbit polyclonal antibody and
the monoclonal antibody 13H1 against vitronectin have been
previously described (Preissner et al. 1987). The monoclonal
10 antibody anti-[VSV-G]-peroxidase and anti-[c-myc]-peroxidase
were obtained from Roche and anti-CD31 monoclonal antibody
EN4 from Monosan. The peroxidase-conjugated rabbit anti-mouse
polyclonal antibody was obtained from Dako.

15 <u>Phage selection procedures</u>
     Phage selections were performed as described with minor
modifications (De Kruif et al. 1995). Briefly, immunotubes
(Gibco BRL) were coated with urea treated, activated
vitronectin (10 μg/ml) overnight at 4°C and blocked with 2%
20 milk powder in PBS (MPBS) for 2 hours. Before the phage were
added to the immunotubes, they were blocked for 15 minutes
with MPBS containing 50% human pooled plasma to absorb non-
specific binding phages and phages recognizing native
vitronectin. The immunotubes were incubated with phages for 2
25 hours at room temperature. After extensive washes with PBS
containing 0.1% Tween-20 followed by washings with PBS alone,
bound phages were eluted with 1 ml 0.1M HCl for 5 minutes at
room temperature. The mixture was neutralized with 500 μl 1 M
Tris-HCl pH 7.4. Eluted phage were used to infect E.coli XL1-
30 blue (Stratagene) and the bacteria were plated on TYE medium
containing the appropriate antibiotics and glucose. After
overnight culture at 37°C, bacterial colonies were scraped
and the bacteria were used to prepare the next library.
     After three rounds of selections, monoclonal phages were
35 prepared from single ampicillin and tetracycline-resistant
colonies of infected XL1-Blue cells. Binding to urea treated

vitronectin was tested in an ELISA using a phage antibody
detection module (Pharmacia, Inc.). A monoclonal anti-M13
antibody (Pharmacia, Inc.) was used and the assay was
performed according to manufacturer instructions. Nucleotide

5   sequence analysis was determined with the ABI PRISM Dye
Terminator Cycle sequencing kit (Perkin Elmer) on an
automated sequencer.

Preparation and purification of scFv fragments

10   The plasmids of the pPVT phagemids were digested with
NotI and NcoI restriction enzymes and the coding regions for
the scFv's were subcloned in the NotI/NcoI digested vector
IM3-HIS that adds 6 histidine residues (HIS-tag) and a myc
tag to the carboxyterminus of the scFv fragment (De Kruif et

15   al. 1996). scFv fragments were produced in the *E.coli* non-
suppressor strain SF110. Periplasmic preparations of scFv's
were purified over a Ni-NTA agarose column (Qiagen) and
eluted by imidazole into 2 ml fractions. The purified scFv
antibody fragments were dialysed against PBS and stored at

20   -20°C until further use.

ELISA's

scFv antibody fragments (30 μg/ml) or a control
polyclonal anti-vitronectin antibody (5μg/ml) were coated

25   overnight on 96 well plates (Nunc Maxisorp). Purified
activated or native vitronectin was allowed to bind for 1
hour at room temperature. After washing with PBS/0.1% Tween-
20, bound vitronectin was determined by incubation with
murine monoclonal antibody 13H1 which recognizes both native

30   and activated vitronectin (Kost et al. 1992). After washing
and incubation with a peroxidase-conjugated rabbit anti-mouse
polyclonal antibody, staining was performed with 2,2'-azino-
di[3-ethyl-benzthiazoline-sulfate] (Roche) and H_2O_2 in 0.05 M
citric acid (pH 4.0). Optical density was read at 405 nm in a

35   microtiter-plate reader (Molecular Devices Corp).

For the competition ELISA, plasma was used as a source
of native vitronectin and serum as a source of activated
vitronectin. Urea activated vitronectin (5 μg/ml) was coated
overnight in a 96 well microtitre plate. Phage or scFv's were
5   pre-incubated with plasma or serum for 30 min. The pre-
blocked phage and scFv's were allowed to bind to coated
vitronectin for 1 hour at room temperature. After washing
with PBS/0.1% Tween-20, binding was determined by incubation
with a peroxidase-conjugated monoclonal anti-M13 antibody
10   (phages) or anti-myc (scFv's). Staining was performed and
measured as described above. The ELISA's were performed in
duplicate or triplicate and repeated at least two times.

Immunohistochemistry

15   Immunohistochemical studies were performed on tissues
from patients with colon-, kidney- or lung cancer and
patients with pneumonia or focal nephro- sclerosis. Also a-
term placental tissue was included. Tissue samples were
immediately frozen in liquid nitrogen after surgical
20   resection and 6 μm thick cryostat sections were prepared.
Analysis was performed after fixation in acetone/0.35% H_2O_2
for 10 minutes. The cryostat sections were air dried for 1
hour and blocked for 1 hour with 4% MPBS/1% BSA. The sections
were subsequently incubated with the scFv antibodies or
25   monoclonal antibody EN4 (áCD31) for 1 hour and washed with
PBS/0.1% Tween-20, followed by incubation for 1 hour with the
peroxidase-conjugated monoclonal antibody anti-VSV-tag or
peroxidase-conjugated rabbit anti-mouse polyclonal antibody.
Anti-thyroglobulin scFv antibody fragment was used as
30   negative control (De Kruif et al. 1995). The conjugate was
developed using diaminobenzidine (Graham and Karnovsky 1966).

Affinity measurements

        The Biacore 2000 system and the reagents, including the
35   sensorchips CM5, surfactant P20 and the amine coupling kit,
containing N-hydroxy-succinimide (NHS), N-ethyl-N'-

(dimethanylamino-propyl)carbidiimide (EDC), ethanolamine hydrochloride and formeate buffer, were from Pharmacia Biosensor AB (Uppsala, Sweden). The whole procedure was performed according to the manufacturers instructions.

5   Briefly, in separate BiaCore flow cells, approximately 7000, 5000 and 2500 resonance units of purified urea treated vitronectin in 10mM formate buffer (pH 3.0) were coupled to a CM5 sensor chip using NHS /EDC amine coupling chemistry. Association and dissociation were measured under continuous

10   flow of 30 μl/min using a concentration range of the anti-vitronectin huMabs from 10 to 100 nM. The association was determined from a plot of (ln(d0/DT)t) versus concentration.

Preparation of endothelial cells

15   Human Umbilical Vein Endothelial Cells (HUVEC) were isolated from umbilical cords using trypsin/EDTA solution, which was applied to the vein using special canules known to persons skilled in the art. The solution was incubated in the vein for 15 min, the detached endothelial cells were flushed out

20   and washed once with RPMI-1640 medium supplemented with a cocktail of general antibiotics and 20% (v/v) of human serum. For the adhesion and apoptosis assay described below, HUVEC's of the second passage were used. Endothelial cells (HUVEC) were brought in suspension with trypsin, trypsin was

25   neutralized in medium containing serum and cells were washed once and resuspended in RPMI-medium containing 1% bovine serum albumin. Cells were counted using a Bürker-Türk chamber and for the adhesion assay 5x10^4 cells were seeded in 96-well plates in the presence of anti-VN antibodies or a control

30   antibody named L1R3.

Adhesion assay

To estimate attached cell numbers, a chromogenic substrate for the ubiquitous lysosomal enzyme, hexosaminidase

35   was used. The protocol for the adhesion assay has been described previously (Landegren 1984). Briefly, urea-treated

vitronectin was coated to wells of a 96 well plate overnight
(5 µg/ml). Plates were blocked with phosphate buffered saline
containing 1 mM calciumchloride, 1 mM magnesium-chloride
(PBS``) and 3% bovine serum albumin (BSA) for 2 hours. HUVEC
5   cells were added to the wells (5x10^4) in the presence of the
antibodies against activated vitronectin or control
antibodies and allowed to adhere for 2 hours at 37°C in a 5%
CO_2 humidified incubator. The plates were washed four times
with PBS``/3% BSA. Hundred µl of 7.5 mM of p-nitrophenol-N-
10   acetyl-β-D-glucoseaminide in 0.1 M citrate buffer, pH 5,
mixed with an equal volume of 0.5% Triton X-100 in water
[NPAG/TX100]) was added to the wells and incubated for 90
minutes at 37°C in a 5% CO_2 humidified incubator. Enzyme
activity was stopped by addition of 150 µl 50 mM glycine
15   buffer, pH 10, containing 5 mM EDTA. Absorbance was measured
at 405 nm in a microtiter-plate reader (Molecular Devices
Corp, Sunnyvale, CA, USA).

Apoptosis assay
20   Annexin-V and propidium iodide were used for apoptosis
measurement. Urea-treated vitronectin was coated overnight to
wells of a 48 well plate (250 µl, 5 µg/ml). HUVEC was added
(5.10^4 cells/well) and allowed to adhere for 30 minutes.
Subsequently, the anti-vitronectin antibodies or a control
25   antibody were added with a concentration of 50 µg/ml and
incubated at 37 °C in a 5% CO_2 humidified incubator. At
different time points the cells were washed and trypsinised.
Apoptosis was determined by flow cytometry using the annexin-
V-fluos staining kit from Roche.

30

Binding assays
        PAI-1 was a gift of Dr Pannekoek (Amsterdam, The
Netherlands) and uPAR from Dr Meijers (Amsterdam, The
Netherlands). A stock solution of 50 µg/ml TAT was formed by
35   incubation of 20 µg/ml with 5 times molar excess of ATIII. No
binding was observed of VN to ATIII alone, so the excess of

ATIII was not removed. Human Collagen type I (Sigma) was
dissolved in 0.5 M acetic acid (1 mg/ml) and further diluted
in PBS. Plasminogen was affinity purified from outdated
plasma using lysine-Sepharose (Pharmacia). The vitronectin

5   ligands PAI-1, plasminogen, uPAR, TAT and collagen type I
were coated overnight to 96-well plates in PBS (5 µg/ml).
Phosphatidyl serine (PS, Sigma) was dissolved in methanol at
a concentration of 50 µg/ml and 50 µl/well was air-dried.
Coated ligands were blocked with PBS containing 3% BSA and

10 0.1% Tween-20 (in the case of PS, Tween-20 was omitted) and
subsequently incubated with urea-treated vitronectin (0-100 µ
g/ml). Binding of vitronectin was determined with a
polyclonal antibody, followed by peroxidase conjugated swine
anti-rabbit IgG and peroxidase substrate (ortho-phenylene-

15 diamine). The concentration that yielded half-maximal
vitronectin binding was calculated. In the competition
experiments, this half maximal concentration of vitronectin
was used and pre-incubated (30 minutes at 37°C) with
increasing amounts of Vn18 IgG or control IgG (0-100 µg/ml).

20 Binding of vitronectin was determined as described above. The
effect of VN18 was expressed as percentage of binding of
vitronectin to its ligands in the presence of control IgG.
For heparin binding experiments, urea-treated vitronectin (5
µg/ml) was coated overnight, blocked and incubated with

25 increasing amounts of biotinylated-heparin (0-100 µg/ml).
Heparin binding was determined with peroxidase-conjugated
streptavidin in the presence of peroxidase substrate. Half-
maximal heparin concentrations were used and different
concentrations of VN18 or control IgG (0-100 µg/ml). Residual

30 heparin binding was expressed as percentage binding of
control IgG.

EXAMPLES

35

Example 1. Identification of phage antibodies that specifically recognize activated vitronectin.

To isolate scFv antibodies against the activated vitronectin phage display on immunotubes coated with urea treated human vitronectin was deployed. It has previously been shown that urea treatment leads to multimerisation of vitronectin, which resembles the conformation of activated vitronectin in serum (Yatohgo et al. 1988). To subtract phage that also bind native vitronectin, phage selections were performed in the presence of plasma as a source of native vitronectin. After three rounds of selection, 48 monoclonal phage antibodies (mophabs) were tested for binding to urea-treated vitronectin by ELISA. One third of the phage were scored positive and selected for further analysis. Nucleotide sequence analysis of the V-regions encoded by these phages unveiled the presence of four independent phage antibodies, which we named Vn1, Vn7, Vn18 and Vn26 (Table I).

Table I. Sequence analysis of monoclonal phage antibodies against activated vitronectin

CDR3 Region	Amino Acid Sequence	
Vn1	CAR - ARRLRLFDY-WGQ	(SEQ. I.D. NO. 1)
Vn7	CAR - DPRGPRSWLDY-WGQ	(SEQ. I.D. NO. 2)
Vn18	CAR - DDRPRELDS-WGQ	(SEQ. I.D. NO. 3)
Vn26	CAR - ANARAAFDS-WGQ	(SEQ. I.D. NO. 4)

It was investigated whether scFv's derived from the
monoclonal phage antibodies specifically recognized activated
vitronectin. scFv's dilutions were used that yielded maximum
binding. Since native vitronectin becomes activated upon
5   coating to plastic, a capture ELISA was performed. A rabbit
polyclonal anti-vitronectin antibody served as a non-specific
control and was coated and used to capture native or
activated vitronectin. As can be seen in Figure 2, scFv(Vn7),
scFv(Vn18) and scFv(Vn26) specifically detected activated
10 vitronectin. Unfortunately, scFv's derived from phage Vn1
could not be analyzed, because the anti-VSV monoclonal
antibody did not detect scFv(Vn1). A possible explanation
could be the presence of a mutation in the sequence encoding
the VSV-tag. As expected, the control polyclonal anti-
15 vitronectin antibody recognized activated and native
vitronectin equally well.

Example 2. Recognition by monoclonal phage antibodies and
scFv fragments of activated vitronectin in serum as compared
20 to native vitronectin present in plasma.

It was investigated whether the anti-vitronectin mophabs
and scFv's recognized activated vitronectin present in serum.
Phage and scFv's were allowed to bind to coated urea-treated
vitronectin in the absence or presence of plasma or serum.
25 As can be seen in Figure 3, binding of all mophabs and scFv's
was inhibited by serum, but not by plasma. These results
strongly suggest that the phages and scFv's also specifically
recognize physiologically activated, serum vitronectin, but
not native, plasma vitronectin.

30

Example 3. Recognition of vitronectin by Vn18 in normal and
tumor tissue sections.

scFv(Vn18) was selected to study the presence and
distribution of activated vitronectin in human carcinoma of
35 colon, kidney and lung, in other pathological specimens, and
in the corresponding normal tissues. Activated vitronectin

was abundantly present in colon adenocarcinoma (Figure 4A),
whereas hardly any activated vitronectin could be detected in
normal colon (Figure 4D). The staining pattern of scFv(Vn18)
overlapped largely with the pattern of staining with

5   antibodies against CD31 (Figure 4B), an endothelial specific
marker, indicating the presence of activated vitronectin
around blood vessels. In addition to the staining around
blood vessels, staining of vitronectin deposits was seen in
the extracellular environment, fibrous tissue, where vessels

10   had not yet been formed.

        In renal cell carcinoma activated vitronectin was mainly
present at the rim of the tumor (Figure 4E). The scFv(Vn18)
staining pattern in these regions is similar to "CD31
staining (Figure 4F), indicating the presence of activated

15   vitronectin around blood vessels. In contrast, in the middle
of the tumor hardly any activated vitronectin was detected,
although "CD31 staining revealed the presence of blood
vessels (data not shown). In regions of the kidney which were
not affected by the tumor hardly any staining for activated

20   vitronectin was seen (Figure 4H). In contrast, the affected
glamorously in kidneys with focal glomerular sclerosis
stained strongly positive (Figure 4N), whereas the normal,
unaffected glamorously were negative (Figure 4O). In non-
small cell lung cancer, similar scFv(Vn18) staining patterns

25   were observed (Figure 4I-K). Finally, in an a-term placenta,
fibrous plaques were positive for activated vitronectin as
well as small capillary-like structures (Figure 4P).

Example 4. Construction and production of a fully human
30   monoclonal IgG$_1$/κ antibody against activated vitronectin.

        Construction of eukaryotic expression vectors and
production of human monoclonal antibodies (huMabs) has been
described in detail elsewhere (Huls et al. 1999). Briefly, in
a two-step cloning procedure, the V$_H$ and V$_L$ regions encoding

35   a scFv were cloned into the vector pLEADER to add the T-cell
receptor α-chain leader HAVT20 leader peptide sequence and a

splice donor site. Next, the V_H and V_L regions, which contain
leader and splice donor sites, were subcloned in the pNUT-Cγ1
or pNUT-Cκ expression vectors. HuMab Vn7, Vn18 and Vn26 were
produced by establishing stably transfected BHK21 cells.

5   Cells were maintained at 37°C in a 5% CO_2 humidified
incubator in Iscove's modified Dulbecco's medium containing
10% FCS, 2 mM glutamine, 100 U/ml penicillin and 100 μg/ml
streptomycin. Recombinant antibodies were purified from
approximately 2 liters of culture supernatant by protein A

10   sepharose affinity chromatography and run on an SDS-PAGE gel
to confirm the stoichiometry and quality of heavy and light
chains as described (Huls et al. 1999). Concentration of
purified huMab was determined by spectophotometry at 280 nm.

15   Example 5. Inhibition of endothelial cell adhesion to
vitronectin.
        For all functional studies, scFv's were converted to
intact, fully human IgG1 monoclonal antibodies by recloning
the V_H and the V_L regions into expression vectors for

20   synthesis of complete IgG_1/κ molecules in BHK cells. Purified
antibodies retained their specificity for activated
vitronectin as assessed in ELISA. The kinetic association and
dissociation rates and affinity constant of the three huMabs
were determined by surface plasmon resonance on a BiaCore.

25   Association and dissociation constants ranged from 1.5×10^5 -
$4.6 \times 10^5 \, M^{-1} s^{-1}$ and 6.8×10^{-4} - $2.9 \times 10^{-2} \, s^{-1}$ respectively, yielding
affinity constants of 4.5, 9.3 and 92 nM for Vn7, Vn18 and
Vn26.
        Vitronectin is believed to play a prominent role during

30   angiogenesis (Preissner et al. 1997). To evaluate the
consequences of binding of human anti-vitronectin antibodies
to activated vitronectin on endothelial function, a number of
assays were performed. First, the effect on endothelial cell
adhesion was analysed. Culture dishes were coated with

35   activated vitronectin and endothelial cells and serial
dilutions of anti-vitronectin antibodies or a control

antibody were simultaneously added to each well. Endothelial
cells (HUVEC's) were allowed to adhere and after washing,
numbers of adhering cells were estimated by adding a
chromogenic substrate for an ubiquitous lysosomal enzyme to
5   the wells (Landegren 1984). Adherence of endothelial cells to
vitronectin-coated plates was inhibited with anti-vitronectin
antibodies in a dose dependent fashion (Figure 5A). All three
anti-vitronectin antibodies inhibited endothelial cell
adhesion (Figure 5B). Maximum inhibition of endothelial cell
10   binding was 80-85% at concentrations of anti-vitronectin
antibodies of 50 µg/ml.

Example 6. Effect of anti-vitronectin antibodies on
endothelial cell apoptosis.
15   Vitronectin protects endothelial cells from entering an
apoptotic pathway (Isik et al. 1998). To evaluate a possible
effect of human anti-vitronectin antibody Vn18 in inducing
endothelial cell apoptosis, anti-vitronectin antibodies or a
control antibody were added to subconfluent layers of
20   endothelial cells (HUVEC's). After 8 hours, cells were
stained with propidium iodide and annexin V and analysed by
flow cytometry. In Figure 6 it is shown that the number of
apoptotic cells increased as a result of binding of the Vn18
antibody to vitronectin-coated plates.
25

Example 7. Effect of anti-vitronectin antibodies on
vitronectin function, mapping of the epitope and cross-
reactivity with vitronectin from other species.
           In addition to a role in cell adhesion, vitronectin is
30   involved in the regulation of protease cascades, such as
coagulation and fibrinolysis (Preissner et al. 1997). To
evaluate a potential effect on these systems the capacity of
anti-vitronectin antibodies to inhibit interactions of
vitronectin with its ligands was determined (Preissner and
35   Seiffert 1998; Schvartz et al. 1999). Binding assays were
performed essentially as described in the materials and

methods. The results are summarized in Figure 7. Vn18 reduced
binding of vitronectin to collagen and inhibited attachment
of endothelial cells almost completely. Binding of six other
vitronectin ligands was not affected.

5

Example 8. Mapping of the epitope on vitronectin that is
recognized by Vn18.

In order to determine the specific epitope for binding
of the antibody to vitronectin, vitronectin fragments as
10   described by Yoneda et al. (1998) were used. Immunoblot
analysis of recombinant vitronectin fragments revealed
binding of Vn18 antibodies to an epitope located in the
hemopexin II domain (Figure 8). For this, several GST-fusion
proteins were purified and let to interact with Vn18
15   antibodies. Domains that are present in vitronectin are S
(aa. 1-42), C (aa. 43-130), H1 (aa. 131-286) and H2 (aa. 287-
459). The subdivision into these four domains is depicted in
Figure 7. The result shown in Figure 8 suggests that Vn18
specifically recognizes a domain near the N-terminus of
20   vitronectin: the H2 domain (aa. 287-459).

Example 9. Cross-reactivity of Vn18 with vitronectin from
other species.

To determine whether Vn18 was also able to recognize
25   vitronectin from other species, vitronectin was purified from
human and chicken serum according to the method of Yatohgo et
al. (1988). An ELISA was performed in which human or chicken
vitronectin (10 µg/ml) were coated onto a 96-wells plate
(Costar), blocked with bovine serum albumin (BSA). Binding of
30   biotinylated IgG of Vn18, a control (phage generated) IgG
(L1R3) or a polyclonal antibody directed against vitronectin
(K9234) was detected with streptavidin-HRP, followed by
staining with a peroxidase substrate. Human and chicken
vitronectin show homology only in the region 268-336 of the
35   hemopexin II (H2) domain (Nagano et al. 1992), indicating

that the epitope for Vn18 is located in this area. Figure 9
shows that Vn18 recognized chicken vitronectin.

Example 10. Effect of VN18 on platelet adhesion and function.

5   Activated vitronectin was sprayed on glass coverslips
and the adhesion of isolated bloodplatelets was studied under
flow. The isolated platelets were reconstituted in 4% human
albumin solution (HAS) to which red blood cells were added,
so no plasma proteins were present. Washed platelets (see

10   below) were resuspended in Hepes-Tyrode buffer (10 mM Hepes,
0.145 mM NaCl, 5 mM KCL, 0.5 mM Na_2HPO4, 1 mM $MgSO_4$,
pH 7.25) to a concentration of 2×10^5 platelets/µl. In the
aggregation assay, 500 µl of this platelet suspension was
pre-warmed to 37°C in the aggregometer for 4 min. Purified

15   IgG of Vn18 or the control IgG L1R3 (see above) were pre-
incubated for 1 min prior to addition of the stimulator
thrombin (0.5 U/ml). Aggregation was monitored for 7 min
under stirring. Figure 10 (left panel) shows that under these
conditions Vn18 caused a concentration dependent inhibition

20   on platelet adhesion, while the maximum concentration of the
control antibody had no effect. In the absence of Vn18, some
platelet aggregates were present. Figure 10 (right panel)
shows that Vn18 addition inhibited mainly the formation of
aggregates, suggesting that Vn18 is able to reduce the

25   activation of platelets. Since this perfusion experiment was
performed in the absence of exogenous vitronectin, Vn18 must
react with vitronectin originating from the platelet
intracellular pool. To investigate this, vitronectin
expression on the platelet membrane was measured in the FACS

30   after mild stimulation of the platelets with thrombin (0.5
U/ml) or epinephrin (100 nM). Platelet rich plasma (PRP) was
obtained from citrated whole blood by centrifugation (10 min
at 200xg, 20°C). One volume of Krebs-Ringer buffer (4 mM KCl,
107 mM NaCl, 20 mM $NaHCO_3$, 2 mM $Na2_2SO_4$, 4.76 mM citric acid,

35   14 mM Na-citrate and 5 mM d-glucose, pH 5.0) was added to one
volume of PRP. The final pH was approximately 6. A platelet

pellet was obtained by centrifugation (10 min at 500xg,
20°C), resuspended in Krebs-Ringer buffer (pH 6.0) and washed
twice by centrifugation (10 min at 500xg, 20°C). After the
second wash, platelets were resuspended in 4% human albumin
5   solution (HAS) (4 mM KCl, 124 mM NaCl, 20 mM NaHCO$_3$, 2 mM Na$_2$
SO4, 2.5 mM CaCl$_2$, 1.5 mM MgCl$_2$, 5 mM d-glucose and 20 U/ml
LMWH, pH 7.35) and incubated at 37°C in the absence or
presence of epinephrin (100nM) or thrombin (0.5 U/ml). In the
FACS analysis, 5x10^5 /well of these platelets were stained
10   using 10 µg/ml biotinylated VN18 or a control (phage display
generated) IgG (L1R3), followed by streptavidin-PE. Figure 11
shows that non-stimulated platelets showed 17.6 % positive
platelets, indicating that the washing procedure already
caused some minor surface expression of platelet-vitronectin.
15   After epinephrin stimulation, this amount increased up to
27%, whereas thrombin stimulation resulted in 47% VN-
expressing platelets. A control antibody (which antibody?)
showed no binding to the platelet membrane. To finally
investigate the effect of Vn18 on platelet aggregation after
20   mild stimulation of the platelets with thrombin (0.5 U/ml),
another aggregation study was performed as described above.
Figure 12 shows that in the presence of 100 µg/ml of Vn18,
platelet aggregation was reduced with 20%, whereas a control
antibody (L1R3) had no effect.
25

    Although the invention has been described with reference
to certain illustrative examples and preferred embodiments,
the scope of the invention is to be determined by reference
to the appended claims.

BRIEF DESCRIPTION OF THE FIGURES

Figure 1. Schematic representation of the activation of
vitronectin and its mediation by a complex of thrombin-
antithrombin III that occurs during pathological processes,
such as angiogenesis.

Figure 2. Specific detection of activated vitronectin by the
scFv fragments Vn7, Vn18 and Vn26 in comparison to a rabbit
polyclonal antiserum that recognizes both activated and
native vitronectin.

Figure 3. A) Monoclonal phage antibody (mophabs) and B) scFv
recognition of activated vitronectin in serum. Phage and
scFv's were allowed to bind to coated urea-treated
vitronectin in the absence or presence of plasma or serum.
Binding of all mophabs and scFv's could be inhibited by
serum, but not by plasma, strongly suggesting that the phages
and scFv's also specifically recognized physiologically
activated, serum vitronectin, but not native, plasma
vitronectin

Figure 4. Immuno histochemistry illustrating the presence of
activated vitronectin in carcinoma of the colon kidney, lung
and other pathological specimens. Also shown are normal
specimens of colon, kidney, lung and brorous tissue which
indicate little activated vitronectin (see text).

Figure 5. A) Binding of endothelial cells to immobilised
vitronectin analysed in the presence of various
concentrations of anti-vitronectin antibody Vn18. B) Binding
of endothelial cells to immobilised vitronectin analysed in
the presence of three anti-vitronectin antibodies (Vn7, Vn18
and Vn26) in a concentration of 50 μg/ml.

Figure 6. Effect of anti-vitronectin antibody Vn18 on endothelial cell apoptosis. The number of apoptotic cells increased from 7.7% to 12.1%.

5 Figure 7. Binding of the indicated ligands on different sections of vitronectin (- no binding; + binding). The effect of Vn18 is expressed as percentage inhibition compared to a control IgG (smb against somatomedin B). Known binding epitopes are presented schematically (top drawing).

10

Figure 8. Immunoblot showing the in vitro interaction of Vn18 with a set of different purified fusion proteins (GST-vitronectin deletion mutants). S, C, H1 and H2 are the different domains in vitronectin (see text and Figure 7).

15

Figure 9. Interaction of Vn18 with chicken vitronectin.

Figure 10. Left panel. Effect of increasing concentrations of Vn18 antibody on platelet adhesion. Right panel. Formation of
20 platelet aggregation in the presence (+Vn18) or in the absence (-Vn18) of the vitronectin specific IgG Vn18.

Figure 11. FACS analysis on Vn18 bound to platelets expressing vitronectin after stimulation by thrombin and
25 epinephrin.

Figure 12. Effect of Vn18 on the formation of platelet aggregates after stimulation by thrombin.

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<120> Activated Vitronectin as a Marker of Angiogenesis
      Detected with Phage Antibodies

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<400> 4
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5   1   5   10   15

CLAIMS

1. An antibody fragment comprising at least a CDR3 domain of
a heavy chain of an antibody which has a higher affinity for
activated vitronectin than for native vitronectin.

2. An antibody fragment according to claim 1 obtainable by
subtractive phage selections on urea-activated vitronectin in
the presence of plasma as a source of native vitronectin.

3. An antibody fragment according to claim 1 or 2, having a
V-region comprising an amino acid sequence of SEQ. I.D. NO.
1, SEQ. I.D. NO. 2, SEQ. I.D. NO. 3 , SEQ. I.D. NO. 4 or a
functional part, derivative and/or analogue thereof.

4. An antibody fragment, comprising at least 60 percent
homology to an amino acid sequence of SEQ. I.D. NO. 1, SEQ.
I.D. NO. 2, SEQ. I.D. NO. 3 , SEQ. I.D. NO. 4 or a
functional part, derivative and/or analogue thereof.

5. An antibody fragment, comprising at least 95 percent
homology to an amino acid sequence of SEQ. I.D. NO. 1, SEQ.
I.D. NO. 2, SEQ. I.D. NO. 3 , SEQ. I.D. NO. 4 or a
functional part, derivative and/or analogue thereof.

6. An antibody fragment according to anyone of claims 1-5,
wherein said vitronectin is human vitronectin.

7. An antibody comprising an antibody fragment of anyone of
claims 1-6.

8. An antibody according to claim 7, which is an IgG_1
antibody.

9. A pharmaceutical composition comprising an antibody
fragment according to anyone of claims 1-6, and/or an
antibody according to claim 7 or 8.

10. A method of diagnosing a disease state in a subject,
said method comprising detecting for the presence of
activated vitronectin in a biological sample taken from said
subject.

11.   The method according to claim 10, wherein the activated vitronectin is detected with use of an antibody and/or an antibody fragment.

12.   The method according to claim 11, wherein said antibody
5   comprises an antibody according to claims 7 or 8, and/or wherein said antibody fragment comprises a fragment according to anyone of claims 1-6.

13.   The method according to claim 11 or 12, wherein said detection of activated vitronectin comprises detecting the
10   presence of said antibody, antibody fragment, or a part thereof, bound to said vitronectin.

14.   An apparatus for detecting the presence of activated vitronectin, derived by selection from a library using a phage display method.

15   15.   A method of detecting the presence of activated vitronectin comprising using an antibody fragment according to anyone of claims 1-6, or an antibody of claim 7 or 8.

16.   Use of an antibody fragment according to anyone of claims 1-6, or an antibody of claim 7 or 8, for the detection
20   of the presence of activated vitronectin.

17.   A use of an activated form of vitronectin as a target for antibody-mediated anti-vascular cancer therapy.

18.   A method of cancer therapy comprising administering anti-activated vitronectin antibody fragments according to
25   anyone of claims 1-6, and/or antibodies according to claim 7 or 8, which interfere with the adhesion and/or migration of tumor-endothelial cells, to a patient.

19.   A method of manufacturing an antibody or an antibody fragment directed toward detecting the presence of activated
30   vitronectin.

20.   An apparatus for detecting the presence of activated vitronectin, using an antibody or an antibody fragment, said antibody or fragment derived by selection from a library comprising urea-treated vitronectin capable of binding to an
35   antibody or antibody fragment of interest, such binding forming a receptor complex and thereby causing a change in a

property associated with the binding partner; and a detection means for detecting the measurable change.

21. The apparatus of claim 20, wherein the receptor complex comprises an antibody or an antibody-binding fragment thereof
5 derived by selection from a library using a phage display method.

22. An isolated peptide fragment comprising at least a CDR3 domain of a heavy chain of an antibody which has a higher affinity for activated vitronectin than for native
10 vitronectin.

23. A peptide fragment according to claim 22 obtainable by subtractive phage selections on urea-activated vitronectin in the presence of plasma as a source of native vitronectin.

24. Use of an antibody according to claim 7 or 8, or an
15 antibody fragment according to anyone of claims 1-6, for at least in part changing an adhesion property of activated vitronectin.

25. A use according to claim 24, wherein said adhesion property comprises the capability to bind to endothelial
20 cells, extracellular matrix proteins, and/or platelets.

26. A use according to claim 25, wherein said changing of the capability to bind to extracellular matrix proteins prevents angiogenesis.

27. A method for at least in part inhibiting adhesion of an
25 endothelial cell to activated vitronectin, comprising providing said vitronectin with an antibody fragment according to anyone of claims 1-6, an antibody according to claim 7 or 8, or a peptide according to claim 22 or 23.

28. A method for at least in part inducing cell death of an
30 endothelial cell, associated with an extracellular matrix comprising activated vitronectin, comprising providing said matrix with an antibody fragment according to anyone of claims 1-6, an antibody according to claim 7 or 8, or a peptide according to claim 22 or 23.

35 29. A method according to claim 28, wherein said cell death comprises apoptosis.

30.   A method for at least in part decreasing an interaction
of activated vitronectin with collagen and/or endothelial
cells, comprising providing said vitronectin with an antibody
fragment according to anyone of claims 1-6, an antibody

5   according to claim 7 or 8, or a peptide according to claim 22
or 23.

31.   An isolated peptide comprising a vitronectin epitope
which is recognized by an antibody fragment according to
anyone of claims 1-6, an antibody according to claim 7 or 8,

10   or a peptide according to claim 22 or 23.

32.   A peptide according to claim 31, which comprises at
least part of the vitronectin H2 domain.

33.   An isolated or recombinant nucleic acid molecule
encoding an antibody fragment according to anyone of claims

15   1-6, an antibody according to claim 7 or 8, or a peptide
according to claim 22 or 23, or a functional part, derivative   .
and/or analogue thereof.

34.   An isolated or recombinant nucleic acid molecule
encoding a peptide comprising a vitronectin epitope which is

20   recognized by an antibody fragment according to anyone of
claims 1-6, an antibody according to claim 7 or 8, or a
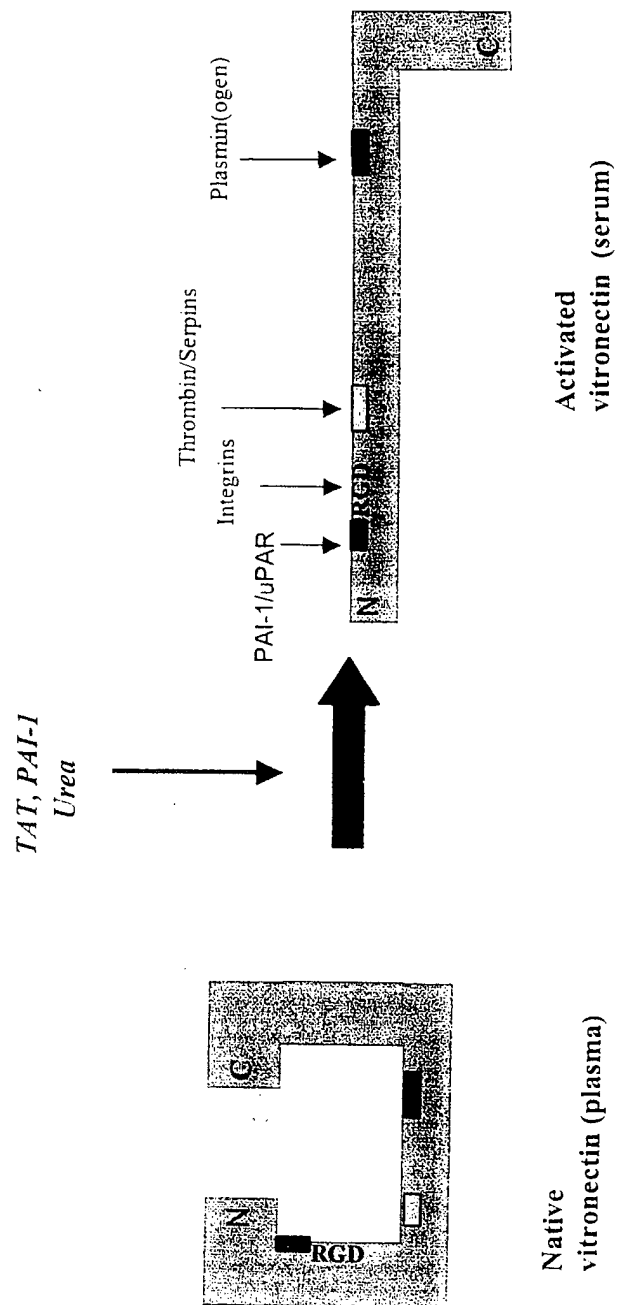peptide according to claim 22 or 23, or a functional part,
derivative and/or analogue thereof.

35.   A nucleic acid molecule according to claim 34, wherein

25   said epitope comprises at least part of the vitronectin H2
domain.

36.   A fusion protein comprising a peptide encoded by a
nucleic acid molecule according to anyone of claims 33-35.

37.   A method for at least in part inhibiting platelet

30   aggregation in the presence of vitronectin, comprising
providing said vitronectin with an antibody fragment
according to anyone of claims 1-6, an antibody according to
claim 7 or 8, or a peptide according to claim 22 or 23.

38.   Use of an antibody fragment according to anyone of

35   claims 1-6, an antibody according to claim 7 or 8, or a

peptide according to claim 22 or 23 for the preparation of a
medicament.

39.   Use of an antibody fragment according to anyone of
claims 1-6, an antibody according to claim 7 or 8, or a

5   peptide according to claim 22 or 23 for the preparation of a
medicament for at least in part inhibiting platelet
aggregation.

40.   Use of an antibody fragment according to anyone of
claims 1-6, an antibody according to claim 7 or 8, or a

10   peptide according to claim 22 or 23 for the preparation of a
medicament for at least in part decreasing an interaction of
activated vitronectin with collagen and/or endothelial cells.

41.   Use of an antibody fragment according to anyone of
claims 1-6, an antibody according to claim 7 or 8, or a

15   peptide according to claim 22 or 23 for the preparation of a
medicament for at least in part changing the development of   .
blood vessels.

42.   A method for at least in part changing the development
of blood vessels, comprising influencing the remodeling

20   process of the extracellular matrix of an endothelial cell.

43.   A method according to claim 42, comprising providing
said extracellular matrix with an antibody fragment according
to anyone of claims 1-6, an antibody according to claim 7 or
8, or a peptide according to claim 22 or 23.

25   44.   An antibody or functional part, derivative and/or
analogue thereof, capable of at least in part inhibiting
binding of an antibody fragment according to anyone of claims
1-6, an antibody according to claim 7 or 8, or a peptide
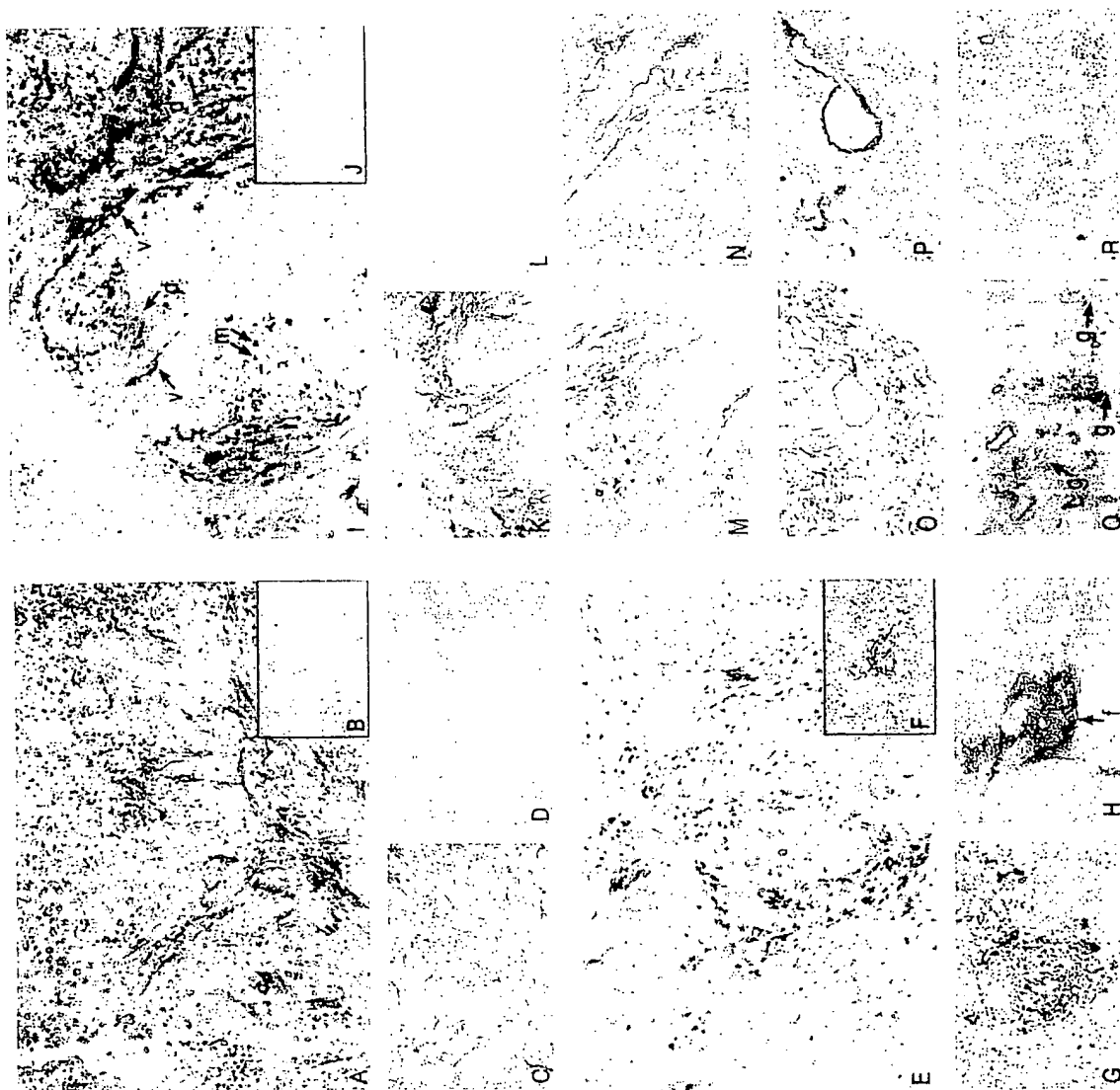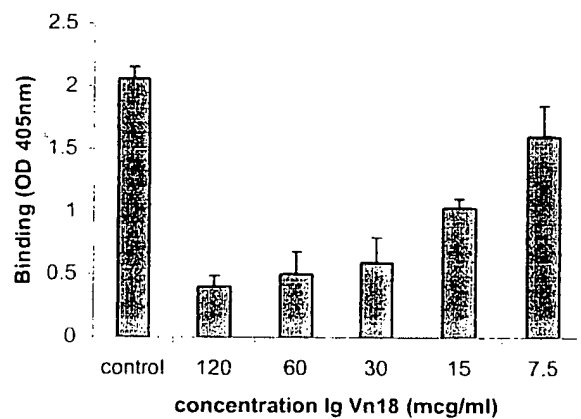according to claim 22 or 23, to activated vitronectin.

Fig. 1

Fig. 2

Fig. 3
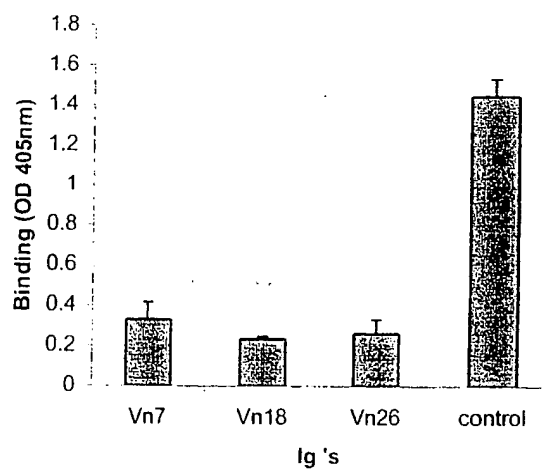
Fig. 4

A

Adhesion of HUVEC on vitronectin +/- Ig Vn18

B

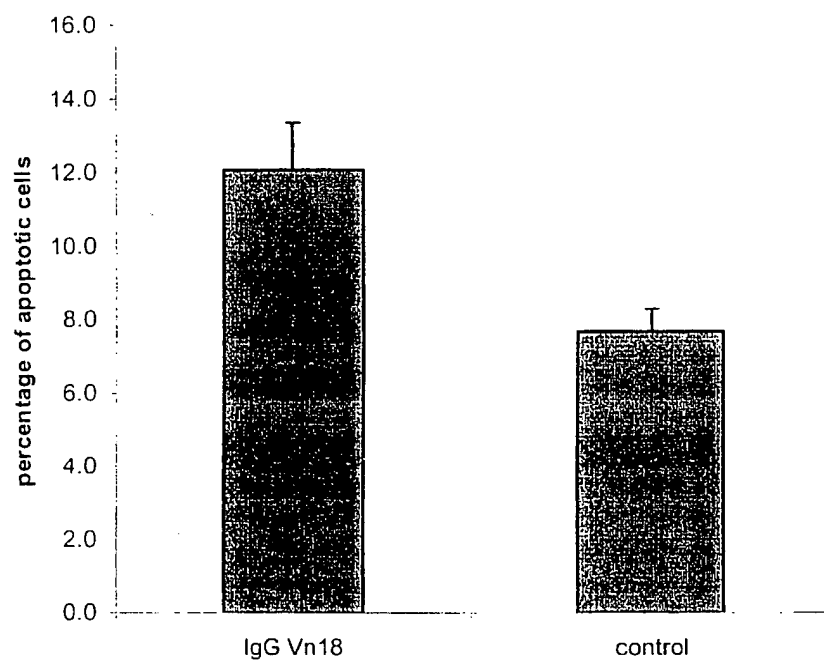**Adhesion of HUVEC on VN +/- Ig Vn**
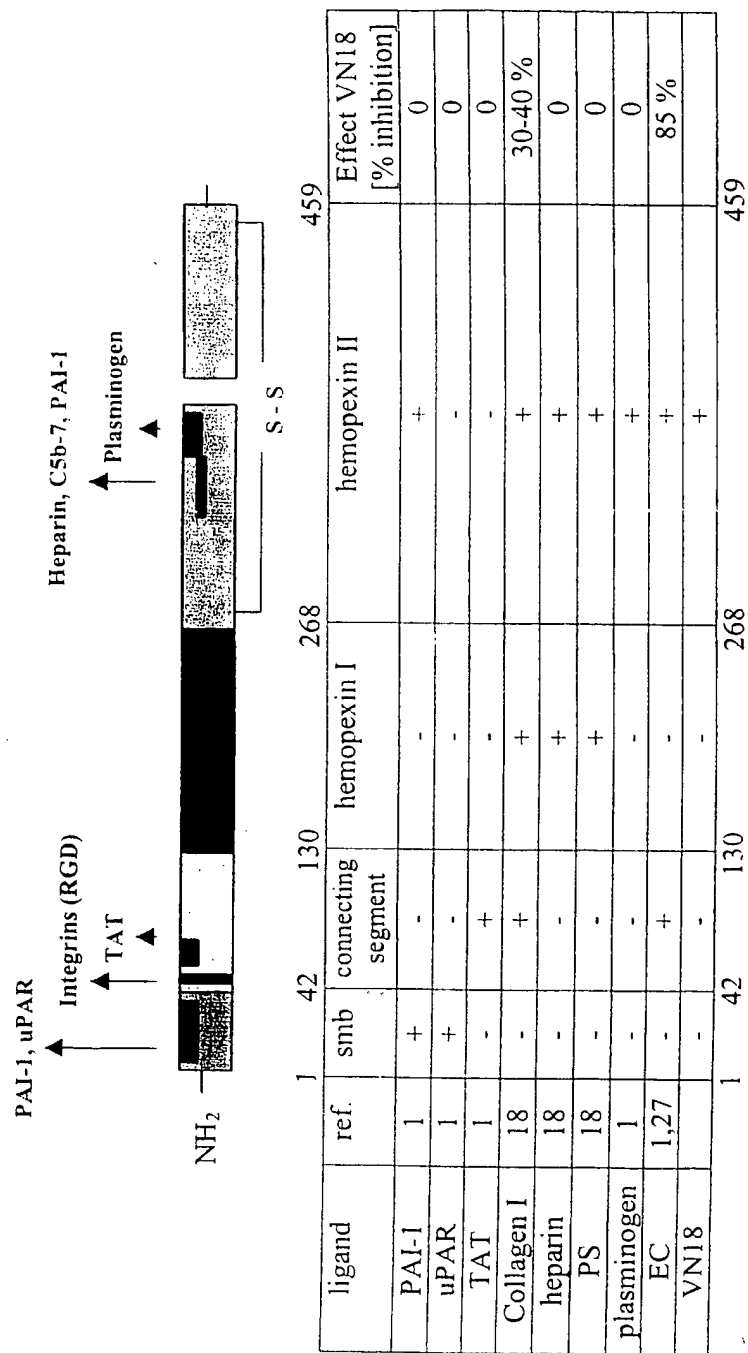

Fig. 5

SUBSTITUTE SHEET (RULE 26)

Fig. 6

Fig. 7

Epitope mapping of Vn18

1 GST + H2
2 GST + H1+2
3 GST + H1
4 GST + SCH1
5 GST + SC
6 GST

Fig. 8

Fig. 9

recognition of human and chicken VN

Legend: BSA, human VN, chicken VN

X-axis categories: L1R3-bio, VN18-bio, K9234-bio

Y-axis: OD [490 nm], scale 0 to 0.6

Fig. 10

Fig. 11